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The Intermediate Subunit of the Gal/Galnac Lectin may Play a Role in Erythrophagocytosis in Entamoeba Histolytica

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THE INTERMEDIATE SUBUNIT OF THE GAL/GALNAC LECTIN MAY PLAY A ROLE IN ERYTHROPHAGOCYTOSIS IN *ENTAMOEBA HISTOLYTICA*

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ABSTRACT

Entamoeba histolytica is a parasite responsible for amebic dysentery and liver abscess in humans. This pathogen causes ~100,000 deaths annually in regions that cannot prevent the fecal-oral route of transmission. Adhesion to host colonic epithelium is a hallmark of infection. The galactose/N-acetylgalactosamine (Gal/GalNAc) lectin is a parasite surface receptor that regulates adhesion to host cells. This lectin is a heterotrimer consisting of Heavy (Hgl), Light (Lgl) and Intermediate (Igl) subunits. Hgl and Lgl are covalently attached to each other by disulfide bonds and interact non-covalently with Igl. Little is known about how the trimer assembles, but lipid rafts, cholesterol- and sphingolipid-rich membrane domains, are thought to be involved. Hgl and Lgl transiently associate with rafts but Igl is constitutively localized to these domains. It is conceivable that Igl, of which there are two isoforms (Igl1 and Igl2), serves to anchor Hgl/Lgl in rafts. We used two techniques to knock down expression of Igl including a short hairpin RNA (shRNA) approach and 'Trigger' approach. Attempts to knock down individual isoforms were not successful. However, when the 'Trigger' approach was used to simultaneously target both isoforms (1A2A cell line), we obtained approximately 57% knock down of Igl2, but not Igl1. These data suggest that Igl1 is essential. The 1A2A cells were able to adhere to and lyse host cells, but could not phagocytose erythrocytes. This implicates Igl2 in erythrophagocytosis. Finally, the submembrane distribution of Hgl/Lgl in the 1A2A cell line was not altered suggesting that Igl2 is dispensable for the association of the dimer with rafts.

DEDICATION

To my parents, John and Anne Rock. The examples you set, the values you instilled, and the expectations you had for me made me the person I am today. Your unconditional love and support make me feel like I can achieve anything I set my mind to. I am grateful for every privilege I have and every opportunity I was given because of you, especially my college education. Thank you for everything. Without you, none of it would be possible.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

I. Introduction

Epidemiological significance

Entamoeba histolytica is a protozoan parasite that is the causative agent of amoebic dysentery and liver abscess. This parasite is prevalent in developing countries that do not have the means to prevent the fecal-oral route of transmission. According to the World Health Organization, 2.4 billion people do not have access to proper sanitation facilities and in some underdeveloped parts of the world, open defecation practices are still being used. This promotes the fecal-oral route of transmission. In 2015, it was estimated that 663 million people worldwide use unsafe drinking water sources, including unprotected wells, springs, and surface water [1], indicating a high risk of infection in these parts of the world. Currently, unsafe drinking water is the biggest factor augmenting diarrheal disease, of which there are 1.7 billion cases a year. Diarrheal disease is the second leading cause of death and a leading cause of malnutrition in children under the age of five [2]. While not all of these cases of diarrhea are due to *E. histolytica*, this pathogen is a major contributor to these statistics. It is estimated that 50 million people worldwide are affected by *E. histolytica* infection, with up to 100,000 deaths annually [3, 4]. This pathogen is also considered a category B bioterrorism agent [5].

Life cycle and pathogenesis

E. histolytica infection occurs after the ingestion of a mature cyst, measuring 10-15 mm in diameter, from fecally-contaminated food or water. The protective chitin cyst wall, which active trophozoites lack, allows the parasite to survive for weeks outside of the host body and to pass through the acidic host stomach unharmed after ingestion. This allows the parasite to travel to the lumen of the small intestine where excystation occurs [6]. The cues that prompt excystation are not well understood [6]; however, studies reveal that *E. histolytica* encodes one active chitinase, *EhCHT1*, which is thought to play a key role in excystation events [7]. Nuclear division occurs in the small intestine, followed by cytoplasmic division to give rise to eight trophozoites, measuring 10-50 mm in diameter. These trophozoites contain a single nucleus with a central karysome [8]. Trophozoites then migrate to the large intestine, where they can multiply by binary fission and produce cysts. The cyst and trophozoite stages are both passed in the feces. It is common for the trophozoites to stay in the intestinal lumen in asymptomatic carriers, who will pass cysts in their stool. Approximately 90% of individuals with an *E. histolytica* infection remain asymptomatically colonized [8]. Alternatively, in symptomatic patients (~10% of infected individuals), the trophozoites colonize the bowel lumen and phagocytose bacteria, erythrocytes, and host cell debris for nutrients. In some patients, the trophozoites invade the colonic epithelium to cause intestinal disease. Amoebic dysentery occurs gradually, and the infected individual will experience symptoms such as abdominal pain and tenderness, and diarrhea, followed by weight loss [8]. Trophozoites can also invade

the bloodstream, liver, brain, and lungs causing extraintestinal disease (Figure 1.1). Liver abscess is the most common manifestation of extraintestinal infections [8].

E. histolytica is closely related to the nonpathogenic *E. dispar*. They were thought to be the same species until 1993 when they were reclassified as distinct species [6]. While they are morphologically identical, they are genetically distinct. Unlike *E. histolytica*, *E. dispar* is nonpathogenic and infection results in asymptomatic colonization of the large intestine [3].

The most common method of diagnosing *Entamoeba* infection is examining stool samples microscopically. Tissue biopsies can also be examined for the presence of trophozoites using light microscopy; however, microscopy techniques alone cannot distinguish between the pathogenic *E. histolytica* and the non-pathogenic *E. dispar*, which are morphologically identical. Antigen and DNA detection methods are also available for diagnosis purposes, as well as noninvasive imaging techniques to visualize abscesses in the liver. In addition to antigen and DNA detection methods, PCR analysis or detection of *E. histolytica*-specific antigens can be used when trying to distinguish *E. dispar* from *E. histolytica* [3].

Current therapies for treating *E. histolytica* infections include luminal amebicides for asymptomatic infections. Metronidazole or tinidazole, DNA synthesis inhibitors, are prescribed for invasive disease, followed by treatment with a luminal agent to completely

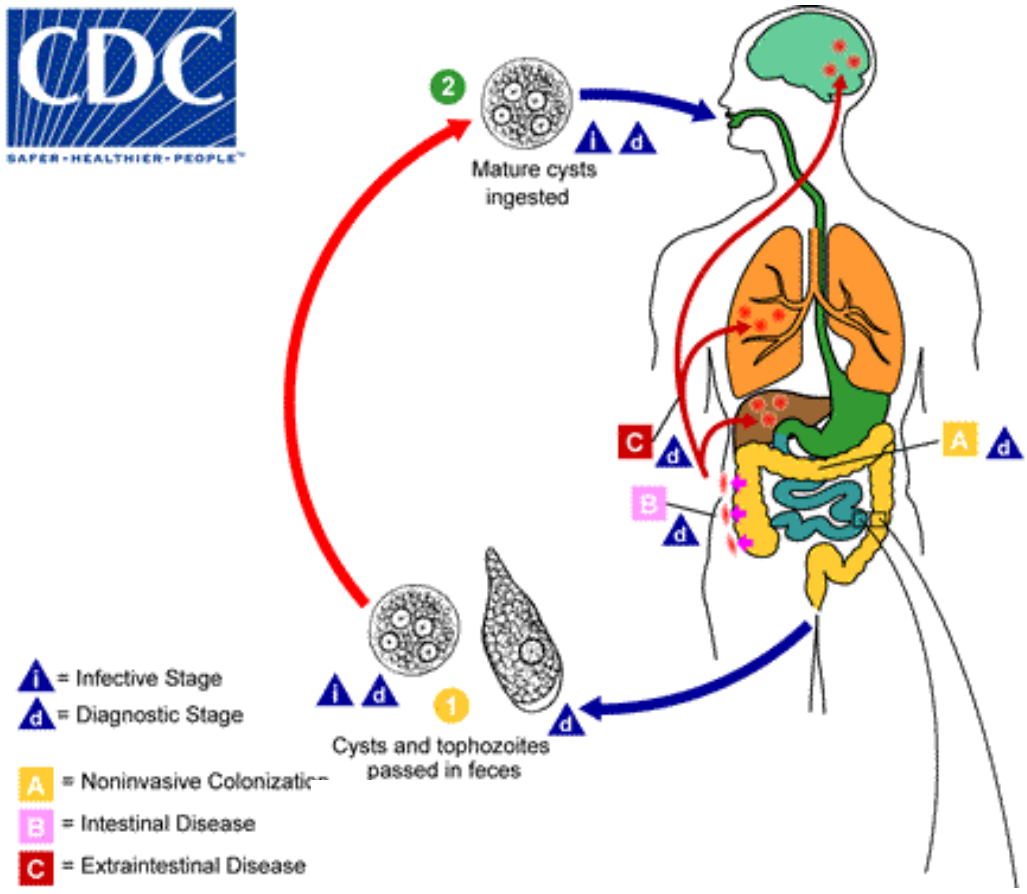

Figure 1.1. Life cycle of *Entamoeba histolytica*

E. histolytica infection occurs after the ingestion of a mature cyst. At this stage in the life cycle, the parasite has a protective cyst wall, which allows it to pass through the acidic host stomach, unharmed, into the small intestines. This is where excystation occurs and trophozoites are released. The trophozoites, which are the active stage of the life cycle, will migrate to the large intestines where they can multiply or produce cysts. Both trophozoites and cysts are passed in the host's stool. Trophozoites can invade the colonic epithelium, causing intestinal disease, and can also cause extraintestinal disease, invading the blood stream, liver, lungs, or brain. Image modified from the Center of Disease Control at https://www.cdc.gov/parasites/amebiasis/pathogen.html.

eliminate infection. In severe cases in patients with amebic colitis, a broad spectrum antibiotic, such as nitroimidazole, also a DNA synthesis inhibitor, can be used and is sometimes used in combination therapy with emetine or dehyroemetine, which are antiprotozoal agents [3]. In addition to drug treatments, a certain emphasis should be placed on prevention, which is limited to environmental and personal hygiene, and can be bolstered by education on health [9]. Prevention is important because the drugs available to treat *E. histolytica* infection often do not result in a 100% clearance of infection.

II. Role of adhesion in virulence

Adhesion to host cells and host extracellular matrix is an important aspect of virulence because the parasite must be able to adhere to host cells in order to cause disease. Adhesion is also the first step in phagocytosis, another important virulence function. There are several amoebic cell surface receptors that mediate parasite-host interactions, including the galactose/N-acetylgalactosamine (Gal/GalNAc) lectin, a 220 kDa membrane protein, L220, a serine-rich *E. histolytica* protein (SREHP), and the adhesion/cysteine protease heterodimer, EhCPADH112 [8].

Amoebic cell surface receptors

L220, is a 220 kDa protein that possesses lectin properties. The protein is localized to the parasite plasma membrane and may be involved in host cell and/or host extracellular matrix attachment. The protein may also serve as the nucleation site for chitin deposition, an important process that occurs during encystation [10]. Outside of having lectin-like

properties, little is known about the function of this protein, or its ligand. However, it is known that the protein is highly immunogenic in mice, hamsters, and rabbits. The purified protein can bind to fixed host cells, thereby blocking the adhesion of trophozoites to the cell monolayer. Antibodies that were prepared against the L220 protein were found to bind to the plasma membrane of trophozoites, partially inhibiting adhesion of the parasite to erythrocytes and other host cells, and inhibiting erythrophagocytosis [11]. This suggests that these antibodies may be useful in the development of vaccines or diagnostic tools [8].

The serine-rich *E. histolytica* protein (SREHP) was identified by a differential cDNA screen that compared the pathogenic *E. histolytica* strain HM1:IMSS to a non-pathogenic strain [12]. This membrane protein, only found in the pathogenic strain of *E. histolytica*, has a high number of post-translational modifications, serine residues, and tandem repeated hydrophilic sequences [13]. Antibodies to SREHP were shown to inhibit adhesion of trophozoites to Chinese hamster ovary (CHO) cell monolayers [12]. It was also found that a SREHP DNA vaccine, administered to mice or gerbils, could provide protection against amebic liver abscess when the animals had undergone a direct hepatic inoculation with trophozoites [14]. More recent work with SREHP has revealed that antibodies to the protein can block Gal/GalNAc lectin-independent uptake of and adhesion to host apoptotic lymphocytes, and to a lesser extent, viable host lymphocytes. Together, these observations strongly implicate SREHP in important virulence functions such as adhesion and phagocytosis [15]. Like the L220 protein, little is known about the

physiological role or ligand of SREHP, but it, too, may prove to have potential as a vaccine target [12].

The 112 kDa heterodimeric protein complex, EhCPADH112, is formed by a cysteine protease, EhCP112, and an adhesin, EhADH112. The complex is localized to the plasma membrane and phagosomes, and antibodies against the complex can limit adhesion, phagocytosis, and destruction of cell monolayers by trophozoites [16]. One study reported that hamsters were protected against liver abscess formation after being inoculated with recombinant EhCP112 and EhADH112 polypeptides [17]. As such, the complex likely regulates important virulence functions and may be a good vaccine target. While EhCPADH112 has been shown to bind to host cells, its specific ligand has not yet been identified [18].

The most well-characterized amoebic cell surface receptor that mediates parasite-host interactions is the Gal/GalNAc lectin. This lectin was originally purified by carbohydrate affinity chromatography [19]. This protein complex binds to galactose and N-acetylgalactosamine residues on host components and is believed to play a vital role in the parasites' ability to adhere to host cells and subsequently cause invasive disease. When trophozoites were exposed to CHO cell glycosylation mutants, which lack Gal/GalNAc-terminal oligosaccharides on their proteins, the parasite was unable to adhere to or kill the host cells [20]. Amebic adherence to CHO cells and human red blood cells (RBCs) was also inhibited when the cells are treated with N-acetyl-$_D$-galactosamine

(GalNAc) because the sugar binds the receptor on the amebic surface, thereby protecting host cells [21]. These studies support the role of the Gal/GalNAc letin in adhesion and invasion. This lectin is a heterotrimer comprised of a heavy subunit (Hgl), intermediate subunit (Igl), and light subunit (Lgl). The heavy and light subunits, 170 and 35 kDa respectively, form covalent dimers connected by disulfide bonds (Hgl/Lgl). The Igl subunit is 150 kDa and is predicted to be glycophosphatidylinositol (GPI)-anchored [22].

The Hgl subunit is a transmembrane protein [22] and is thought to be the key molecule in the parasite's adherence because it possesses a carbohydrate recognition domain (CRD) on its exoplasmic domain [23]. Hgl also has a short cytoplasmic tail that may be involved in intracellular signaling because it has sequence identity with the cytoplasmic tails of β2 and β7 integrins. Integrins are a well-studied family of mammalian adherence proteins. The cytoplasmic tails of integrins regulate interaction with host cytoskeleton and signal transduction. The sequence of the cytoplasmic domain that Hgl shares with β2 and β7 integrins includes amino acids that are implicated in control of integrin-actin adhesiveness [24], suggesting that the molecular mechanisms that regulate adhesion by the integrins and Hgl may be similar.

To gain insight into the function of Hgl domains, several studies have characterized cell lines that express truncated forms of Hgl [24, 25, 26, 27]. In one such study, two cell lines were created that expressed the cytoplasmic tail of the Hgl subunit fused to green fluorescent protein (GFP). In the first cell line (224), the lectin portion of the fusion

protein consisted of the amino-terminal signal peptide, the putative transmembrane domain, and the cytoplasmic tail of Hgl. In the second cell line (324), the lectin portion of the fusion protein consisted of the amino-terminal signal peptide and the putative transmembrane domain, but lacked the cytoplasmic tail. In both cases, expression of the fusion protein was inducible by the addition of tetracycline to the medium. Immunofluorescence staining with an anti-GFP antibody and confocal microscopy revealed that fusion proteins 224 and 324 were not localized to the cell surface, but instead, were found in puncta within the cytoplasm suggesting a vesicular location. When amebae were induced to express the 324 fusion protein, adhesion to CHO cells was unchanged from that of the control. Alternatively, when amebae were induced, to express the 224 fusion protein containing the cytoplasmic domain, there was a dominant negative effect characterized by a decrease in the parasites' ability to adhere to CHO cells. To complement these *in vitro* studies, 224 and 324 transgenic trophozoites were inoculated intrahepatically into gerbils, and expression was induced by adding doxycycline to the gerbils' drinking water. There was no significant difference in liver abscess size in gerbils infected with the 324 cell line before or after induction of expression. However, gerbils infected with the 224 cell line exhibited abscesses that were 84% smaller in the induced condition versus the non-induced condition [24].

In another study, a cell line was generated that expressed a truncated Hgl, named HGL-2, in which the extracellular domain was replaced by a FLAG epitope. Trophozoites expressing the fusion protein exhibited a reduction in adhesion to host (Caco-2) cell

monolayers and reduced motility. One possible explanation is the cytoplasmic tail of the fusion protein interacts with and sequesters intracellular factors, and in doing so, disables the function of the endogenous subunit [25]. The HGL-2 cell line was also less virulent when injected into hamsters intraportally. Specifically, HGL-2–expressing trophozoites produced a high number of hepatic foci but these were smaller than those in hamsters infected with control trophozoites. It was also found that in hamsters infected with HGL2-expressing trophozoites fewer macrophages were recruited to the site of infection and there were few apoptotic or necrotic host endothelial cells [26]. Interestingly, trophozoites expressing HGL-2 could still lyse host cells when incubated *ex vivo* with human colonic tissue segments. A histological assay of the explants also determined that the HGL-2 cell line could initiate the tissue inflammatory process [27].

The 35 kDa Lgl subunit of the Gal/GalNAc lectin, which is predicted to be glycophosphatidylinostitol (GPI)-anchored, has also been shown to play a role in virulence [28, 29]. It, itself has no carbohydrate binding capability and removal of the GPI-anchor prevents its association with Hgl [30]. In one study, expression of Lgl was inhibited by antisense RNA. Trophozoites with reduced expression of Lgl did not exhibit an adhesion defect to host cells, but their cytopathic and cytotoxic activities and their ability to cause liver abscess in hamster was strongly inhibited. This was the first study implicating Lgl in amoebic virulence [29].

Cells expressing a truncated version of the Lgl, in which the 15 C-terminal amino acid residues were deleted, were unable to form a heterodimer with Hgl. This effect may be due to the deletion of the GPI addition site that is predicted to be in the deleted sequence [31]. Cells expressing a truncated version of the Lgl, in which the N-terminus was removed, displayed a significant decrease in their ability to adhere to and phagocytose erythrocytes [28]. Capping is a process by which the parasite's bound surface receptors cluster into small patches at the posterior pole of the cell, to form a structure known as the uroid. As the parasite moves, the uroid is shed from the parasite surface. Because capping can result in sequestration and shedding of host antibodies at the uroid, the process is thought to be another important virulence function involved in evasion of the host immune response [32]. Transfected cells expressing N-truncated Lgl also had a significantly reduced ability to cap the surface Gal/GalNAc lectin to the uroid region. These results further implicate the light subunit, and specifically the N-terminal domain, in virulence [28].

There are five isoforms of Lgl, *Lgl1* to *Lgl5*. These five genes have high sequence homology, but the most pronounced difference between the first group, Lgl1 to Lgl3, and the second group, Lgl4 and Lgl5, is a deletion of 17 amino acids in the second group [33]. The similarity shared among the isoforms has confounded silencing attempts. In one study, when the expression of the *Lgl1* gene was transcriptionally silenced by transgene-induced silencing, there was simultaneous down-regulation of the expression of the closely related *Lgl2* and *Lgl3* genes. This strain was called RBV. Simultaneous silencing

of *Lgl1, 2* and *3*, significantly reduced the trophozoites' ability to cap surface Gal/GalNAc lectin to the uroid region [30]. Despite silencing of all three genes and the capping defect, RBV trophozoites were able to invade the colon mucosal surface and trigger an inflammatory response when incubated with human colon explants [27]. Interestingly, expression of Lgl4 and Lgl5 was up-regulated in the RBV cell line. Likewise, a second cell line, L5, in which expression of Lgl5 (and consequently also Lgl4) was silenced by transgene-induced silencing, expression of Lgl1-3 was upregulated [33]. The simultaneous silencing of Lgl4 and Lgl5 in the L5 cell line had no effect on the ability of trophozoites to cap the Gal/GalNAc lectin complex or take up erythrocytes by phagocytosis [33], suggesting that the Lgl4 and Lgl5 subunits are not essential for these processes. Attempts to silence all five isoforms of the Lgl subunit at once were not successful, indicating the possibility that silencing the entire family of *Lgl* genes may be lethal for the trophozoites [33].

The 150 kDa Igl subunit of the Gal/GalNAc lectin is thought to be GPI-anchored and to non-covalently interact with the Hgl/Lgl heterodimer. It was first identified as a trophozoite surface antigen. Interestingly, a monoclonal antibody (EH3015) that recognized this surface antigen also reacted with the 170 kDa Hgl subunit of the Gal/GalNAc lectin in a different strain of *E. histolytica* (H-302:NIH) [34]. Hamsters that were immunized with the purified antigen target of EH3015, and subsequently inoculated intrahepatically with trophozoites, developed significantly smaller liver abscesses than the control, while some hamsters were completely protected from abscess formation [35].

Trophozoites adhered poorly to CHO cells that were pretreated with the same purified antigen (mentioned above) [34]. Together, these studies gave the first indication that the 150 kDa surface antigen might also be a vaccine candidate.

To clarify the relationship between Igl and Hgl immunofluorescence staining and confocal microscopy were used to assess colocalization. These studies revealed that Igl colocalized with the Hgl/Lgl heterodimer in the plasma membrane, suggesting it plays a cooperative role in host-parasite interaction [36]. Several other lines of evidence have been cited to support the notion that Igl is part of the Gal/GalNac lectin: (1) there was a mAb (EH3077) that recognized both Igl and Hgl, suggesting the proteins may share a common epitope, (2) the mAb EH3015, specific to the 150 kDa surface antigen, also reacted with Hgl in the H-302:NIH strain and when antigen recognized by this mAb was purified from the H-302:NIH stain, the SDS-PAGE pattern was the same as the pattern from the HM-1:IMSS strain, (3) the same electrophoretic pattern of proteins purified by immunoaffinity chromatography resulted using mAb3231 (antibody specific to Hgl in HM-1:IMSS strain) and mAb EH3015 (antibody specific to Igl in HM-1:IMSS strain), and (4) Western blotting analysis showed that the Hgl/Lgl heterodimer was recognized by mAbs EH3015 and EH3033 (antibody specific to Hgl in HM-1:IMSS strain), suggesting that the Hgl and Igl subunits may exist in dimers under native conditions [34]. Simple cross-reactivity of antibodies is not sufficient to conclude that Igl is part of the Gal/GalNAc lectin, nevertheless, it is generally accepted that Igl is part of this complex.

Southern blots have provided evidence that there are two isoforms of Igl (Igl1 and Igl2), which have 81% sequence homology [36]. Confocal microscopy shows Igl1 and Igl2 are localized on the plasma membrane and in the cytoplasm of trophozoites. Both isoforms were found to be colocalized in most cells, although different localization patterns were seen on vacuoles in the cytoplasm [37]. Expression of the Igl1 gene is seven times higher than expression of the Igl2 gene as determined by real-time PCR [38]. While the primary structures of the two isoforms of Igl are similar, it may be possible that these two Igls have different functions and different levels of expression during phagosome maturation [37]. Unlike the Hgl subunit, Igl does not have a CRD. A BLAST search revealed that the Igl sequence contains multiple CXXC and CXC sequence motifs [36], bearing a resemblance to the variant surface glycoproteins (VSPs) of *Giardia lamblia*, which play a role in host immune evasion. The high number of cysteine residues in Igl may be important for the maintenance of their active conformations [37].

In a more recent study, the effect of mAbs on amebic adherence and liver abscess formation was observed. Four different mAbs were used: XEhI-20, specific to Igl1, XEhI-28, specific to the N-terminal of Igl1, XEhI-B5, specific to Igl2, and XEhI-H2, specific to the middle part and C-terminal of both Igl1 and Igl2. When CHO cells were pretreated with the mAb, XEhI-28, there was no effect on adhesion, while the other three mAbs significantly inhibited amebic adherence. The inhibitory effect of XEhI-B5 was significantly better than that of XEhI-20 and XEhI-H2, and the inhibitory effect of XEhI-H2 was significantly better than that of XEhI-20. A hamster model was used to study the

effects of the mAbs on liver abscess formation. Hamsters were passively immunized with the three mAbs that inhibited adhesion to CHO cells. XEhI-20-innoculated hamsters had the lowest mean percentage of abscessed liver, and XEhI-H2-innoculated hamsters had the highest mean percentage of abscessed liver. However, all three mAbs conferred some protection when compared to hamsters immunized with isotype controls. This suggests that both Igls must be involved in adhesion, but because XEhI-28 did not affect adhesion to CHO cells, the N-terminus of Igl1 may not be important for adhesion to host cells [37].

Igl may also have hemagglutinating, hemolytic, and cytotoxic activities, which reside in the C-terminal region of the protein [23]. Recombinant full-length Igl (F-Igl) or fragments of Igl corresponding to the N-terminal (N-Igl), middle (M-Igl), and C-terminal (C-Igl) regions were incubated with horse red blood cells (HoRBCs) or human red blood cells (HuRBCs). All versions of Igl were able to hemagglutinate RBCs but only the C-terminal fragment was able to hemolyse RBCs. Incubating trophozoites with mAbs XEhI-28 and XEhI-H2 significantly inhibited hemolytic activity. Hemolytic activity was inhibited the most with the XEhI-H2 mAb, which is specific to the middle/C-terminal part of Igl, supporting the idea that the C-terminal region of Igl is responsible for the main hemolytic activity. The recombinant fragments of Igl were also incubated with Caco-2 cells to test cytotoxic activity. F-Igl and C-Igl proteins attached to the cells, causing cell death, indicating that the C-terminal region of Igl is also cytotoxic [23].

Diagnosing *E. histolytica* infection presents challenges because of the closely related, and morphologically identical *E. dispar*, and the high number of asymptomatic patients. One study indicates that Igl, especially the carboxyl (C)-terminal portion, is well recognized in both symptomatic and asymptomatic patients with *E. histolytica* infections and is useful for the serodiagnosis of amebiasis. Full length of Igl (F-Igl) and three partial fragments of Igl (N-terminal part, middle part, and C-terminal part) were used as antigens for an enzyme-linked immunosorbent assay (ELISA) to characterize the antibodies present in the sera from patients with amebic liver abscess or amebic colitis, asymptomatic cyst passers, individuals with other protozoan infections, and healthy controls. The C-terminal part of Igl was the most sensitive (97%) and the most specific (99%, with only one false-positive out of 162 serum samples) to *E. histolytica* antibodies in the patient sera, the middle part of Igl showed 92% sensitivity and 99% specificity to *E. histolytica* antibodies in the patient sera, the full length Igl showed 90% sensitivity and 94% specificity, and the N-terminal part of Igl, which was only 56% sensitive, with 96% specificity, indicating the C-terminal part of Igl is the best for serodiagnosis of amebiasis [39].

Igl is predicted to be GPI-anchored to the plasma membrane. Cell lines were created using antisense RNA to knock down expression of the GlcNAc-phosphatidylinositol deacetylase (PIG-L) gene. These cell lines showed a reduction in GPI-containing proteins, as demonstrated by fluorescence imaging with Alexa 488-labeled proaerolysin (FLAER), a GPI-anchor specific stain. These cell lines also exhibited a growth defect, a decrease in fluid phase pinocytosis, and a reduction in adhesion to target cells, supporting

the theory that GPI-anchored molecules are involved in pathogenesis. However, subunits of the Gal/GalNAc lectin are not the only GPI-anchored proteins found in *E. histolytica*; proteophosphoglycans and undoubtedly other proteins, are also affected by the reduction of GPI-anchors and could contribute to the phenotype seen in the mutant cell lines [40].

Knocking down the expression of a protein in trophozoites is a good way to determine the role of that protein in the cell. It was reported that the expression of Igl can be knocked down using a short hairpin RNA (shRNA) approach [41]. Four different shRNAs were used to knock down expression. One of the constructs targeted only Igl1, while three of the constructs targeted both Igl1 and Igl2. The level of knock down was measured by Western blotting and ranged from 4.7% to 72.2% reduction in expression. However, these cell lines were never further characterized for the effect of the knock down on various virulence functions [41] or on Gal/GalNAc lectin assembly.

Lipid rafts and their role in adhesion

Lipid rafts are specialized membrane domains that are highly ordered, tightly packaged, and enriched in cholesterol, glycosphingolipids, and phospholipids [42]. They are referred to as detergent-resistant membranes (DRMs) because they are detergent insoluble. In *E. histolytica*, raft-like microdomains are known to play an important role in adhesion to host cells, pinocytosis, and secretion [43]. When trophozoites were treated with methyl-β-cyclodextrin (MBCD), a cholesterol binding agent, adhesion to CHO cell monolayers, collagen, and fibronectin, as well as pinocytosis were significantly inhibited,

indicating these processes rely on the presence of cholesterol [43, 44]. Contrastingly, the ability of the cells to secrete cysteine proteases was not significantly inhibited, indicating that this process is not dependent on rafts [43]. The inhibition of adhesion by treatment with a raft-disrupting agent suggests that adhesion molecules, like the Gal/GalNAc lectin may localize to rafts. This was tested by isolating rafts by detergent-extraction and sucrose gradient ultracentrifugation and analyzing fractions using SDS-PAGE and Western blotting with antibodies specific to the Hgl subunit and Lgl subunit. It was found that fractions 9 through 13 contained the raft-like region of the sucrose gradient [43]. Under certain conditions, the Hgl/Lgl heterodimer becomes enriched in lipid rafts. Such conditions, referred to as "stimulation," include loading parasites with cholesterol or exposing them to host cells or host extracellular matrix. Although the levels of Hgl/Lgl vary in rafts depending on conditions, the Igl subunit is constitutively localized to lipid rafts under all known conditions [22]. Therefore, when trophozoites are stimulated, the three subunits become colocalized in the lipid raft membrane domains (Figure 1.2). This condition is correlated with an increased ability of the parasite to bind to host cells in a galactose-specific manner [45]. Biotinylation of cell surface proteins indicate there is not a significant increase in the lectin level on the cell surface after cholesterol loading, suggesting that the Hgl and Lgl subunits may move laterally into rafts upon stimulation [45]. However, the cycling of the lectin to and from the cell surface cannot be completely ruled out. While there is much known about these subunits, there is still a considerable amount to be learned about the mechanism by which the lectin assembles into a functional complex.

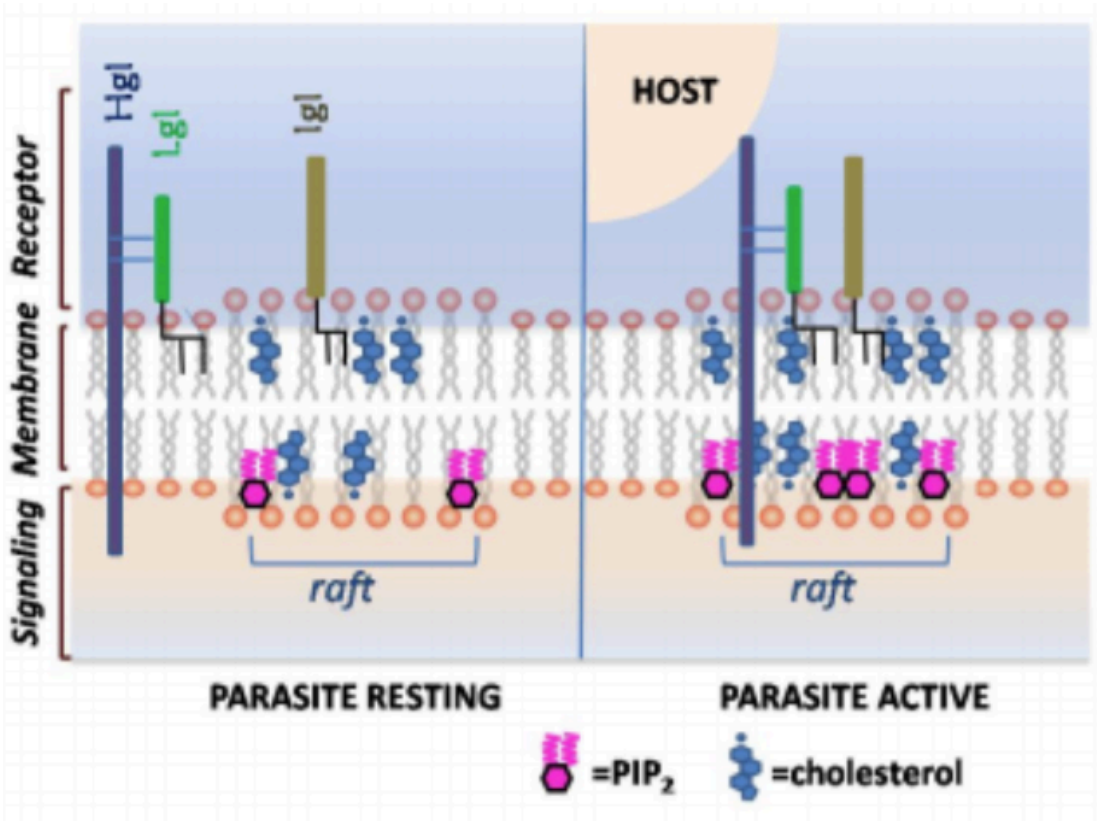

Figure 1.2. Proposed submembrane distribution of the subunits of the Gal/GalNac lectin when parasite is resting versus active

Under all known conditions, Igl is predominately localized in lipid raft membrane domains. In the absence of host components (shown on the left), the majority of the Hgl/Lgl heterodimer is localized to non-raft membrane. Under certain conditions, such as loading parasites with cholesterol or exposing them to host cells or host extracellular matrix, the parasites are activated (shown on the right) and there is an enrichment of the Hgl/Lgl heterodimer in lipid rafts [22]. The colocalization of the three subunits in raft membrane domains correlates with an increased ability of the parasite to adhere to host cells in a galactose-specific manner [45]. The constitutive localization of the Igl subunit in rafts would suggest that it regulates the submembrane distribution of the Hgl/Lgl heterodimer.

IV. Summary

The ability of *E. histolytica* to cause infection is dependent on the trophozoites' ability to adhere to host cell and host extracellular matrix. Lipid rafts are an important factor in the adhesion process. Disruption of rafts inhibits the trophozoites' ability to adhere to host cells [43, 44], and co-localization of all three subunits in rafts during stimulation support this hypothesis. The constitutive localization of Igl to rafts under all conditions is intriguing and **we hypothesize that Igl forms an anchor that facilitates interaction of Hgl/Lgl in rafts.** Therefore, Igl and its role in Gal/GalNAc lectin assembly is the focus of this study. That Igl may also regulate hemolysis [23], an important virulence function, further supports the need to study this protein. Despite apparent successful knock down of Igl using shRNA [41], the characterization of such a cell line has not been undertaken. Because of its role in adhesion, the Gal/GalNAc lectin is a possible drug, vaccine, and diagnostic target. This knowledge could lead to important discoveries for diagnosing and treating *E. histolytica* infection, therefore a better understanding of how the Gal/GalNAc lectin assembles into an active complex is necessary. Accordingly, the aims of this study were:

1. To determine if Igl facilitates the enrichment of Hgl/Lgl in rafts.
2. To determine if Igl plays a role in virulence.

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CHAPTER 2

THE INTERMEDIATE SUBUNIT OF THE GAL/GALNAC LECTIN MAY PLAY A ROLE IN ERYTHROPHAGOCYTOSIS IN *ENTAMOEBA HISTOLYTICA*

I. Abstract

Entamoeba histolytica is a parasite responsible for amebic dysentery and liver abscess in humans. This pathogen causes ~100,000 deaths annually in regions that cannot prevent the fecal-oral route of transmission. Adhesion to host colonic epithelium is a hallmark of infection. The galactose/N-acetylgalactosamine (Gal/GalNAc) lectin is a parasite surface receptor that regulates adhesion to host cells. This lectin is a heterotrimer consisting of Heavy (Hgl), Light (Lgl) and Intermediate (Igl) subunits. Hgl and Lgl are covalently attached to each other by disulfide bonds and interact non-covalently with Igl. Little is known about how the trimer assembles, but lipid rafts, cholesterol- and sphingolipid-rich membrane domains, are thought to be involved. Hgl and Lgl transiently associate with rafts but Igl is constitutively localized to these domains. It is conceivable that Igl, of which there are two isoforms (Igl1 and Igl2), serves to anchor Hgl/Lgl in rafts. We used two techniques to knock down expression of Igl including a short hairpin RNA (shRNA) approach and 'Trigger' approach. Attempts to knock down individual isoforms were not successful. However, when the 'Trigger' approach was used to simultaneously target both isoforms (1A2A cell line), we obtained approximately 57% knock down of Igl2, but not Igl1. These data suggest that Igl1 is essential. The 1A2A cells were able to adhere to and lyse host cells, but could not phagocytose erythrocytes. This implicates Igl2 in erythrophagocytosis. Finally, the submembrane distribution of Hgl/Lgl in the 1A2A cell

line was not altered suggesting that Igl2 is dispensable for the association of the dimer with rafts.

II. Introduction

Entamoeba histolytica is a protozoan parasite that is the causative agent of amoebic dysentery and liver abscess in regions of the world that cannot properly sanitize their water in order to stop the fecal-oral route of transmission [1]. It is estimated that worldwide, there are 50 million people infected by this parasite annually, causing up to 100,000 deaths a year [2, 3]. In addition, this pathogen is also considered a category B bioterrorism agent [4]. *E. histolytica* infection occurs by the ingestion of mature cysts in fecally-contaminated food or water. The protective cyst wall allows the parasite to survive outside of the host body for weeks and after ingestion, to pass through the acidic conditions of the host stomach unharmed. Excystation occurs in the small intestine and trophozoites are released. Trophozoites migrate to the large intestine, where they can multiply by binary fission and produce cysts; both stages are passed in the feces. It is common for the trophozoites to stay in the intestinal lumen in asymptomatic carriers, who will pass cysts in their stool. In symptomatic patients, the trophozoites colonize the bowel lumen and lyse and/or phagocytose bacteria, erythrocytes, and host cell debris for nutrients. In some patients, the trophozoites invade the colonic epithelium to cause intestinal disease. However, trophozoites can also invade the bloodstream, liver, brain, and lungs causing extraintestinal disease [1].

Adhesion to host cells and host extracellular matrix is an important aspect of virulence, because the parasite must have the ability to adhere to host cells in order to cause disease. The most well-characterized amoebic cell surface receptor that mediates parasite-host interactions is the galactose/N-acetylgalactosamine (Gal/GalNAc) lectin [5]. This protein complex binds to galactose and N-acetylgalactosamine residues on host components and is believed to play a vital role in the parasites' ability to adhere to host cells and invade the colonic epithelium [6]. The Gal/GalNAc lectin is composed of a Heavy subunit (Hgl), Intermediate subunit (Igl), and Light subunit (Lgl). The heavy and light subunits are connected with disulfide bonds and are thought to interact non-covalently with the intermediate subunit [5]. The transmembrane Hgl subunit is thought to play a key role in adhesion because its exoplasmic domain contains a carbohydrate recognition domain. The cytoplasmic tail of Hgl may be involved in intracellular signaling because it has sequence identity with the cytoplasmic tails of β2 and β7 integrins [7]. The Lgl subunit, which is proposed to be GPI-anchored, has been shown to have cytotoxic and cytopathic activity and to play in important role in capping the surface Gal/GalNAc lectin to the uroid region [8,9]. The Igl subunit, which is also proposed to be GPI-anchored, is implicated in adhesion, and is thought to have hemagglutinating, hemolytic, and cytotoxic activities [10]. Of the three subunits, the least is known about Igl and the role it plays in virulence.

Lipid rafts are cholesterol, glycosphingolipid, and phospholipid-rich membrane domains. In *E. histolytica*, these regions of the membrane are known to play an important role in

adhesion to host cells, pinocytosis, and secretion [11]. Under certain conditions the Hgl/Lgl heterodimer move into lipid rafts. Such conditions, referred to as “stimulation”, include loading parasites with cholesterol or exposing them to host cells or host extracellular matrix. On the other hand, Igl is constitutively localized to lipid rafts, under all known conditions. Colocalization of the three subunits in the lipid raft regions is correlated with an increased ability of the parasite to bind to host cells in a galactose-specific manner [12]. However, the assembly of the subunits of the Gal/GalNAc lectin into an active complex in rafts is not fully understood.

The constitutive localization of Igl to rafts suggests that it regulates Hgl and Lgl interactions with rafts. However, a comprehensive study of Igl has never been undertaken. Therefore, in this study, we aimed to knock down the expression of Igl and characterize the cell lines with reduced expression of Igl. Such characterization should provide insight into the role of Igl in virulence and in regulating Gal/GalNAc lectin-raft interactions.

III. Materials and Methods

Cell culture conditions

E. histolytica trophozoites (strain HM-1:IMSS) were cultured axenically in TYI-S-33 media in 15 ml glass screw cap tubes or T25 cell culture flasks at 37°C [13].

Antibody development

The polyclonal Hgl antibody was developed commercially (Thermo Scientific, Waltham, MA) in rabbits against the synthetic polypeptide- CQKEYAYPIEKYEVDWDNVPVDE targeting the Hgl levels. These antibodies were purified by ELISA and confirmed against the synthetic polypeptide used in the initial immunization.

Plasmid construction

Plasmids (pGIR310) encoding shRNAs directed against green fluorescent protein (GFP; control) or against two different sequences within Igl1 were generously provided by Dr. W.A. Petri, Jr. (University of Virginia School of Medicine, Charlottesville, VA) [14]. The two shRNA constructs directed against Igl corresponded to the codons for amino acids 272-300 in Igl1, Igl(272-300), or to the codons for amino acids 1198-1226, Igl(1198-1226), which is a sequence conserved in both Igl1 and Igl2 [14]. All pGIR310-based plasmids contained the hygromycin-resistance gene for selection.

To construct the plasmids for trigger-mediated knock down of expression, the gene encoding Igl1 was isolated by PCR using purified (Wizard Genomic DNA Purification Kit, Promega) *E. histolytica* genomic DNA as template. Due to the large size of the *Igl1* gene (3,352 nucleotides), the first and second halves of the *Igl1* gene were amplified by PCR separately. The cDNA encoding the entire *Igl2* gene was commercially synthesized (GenScript, Piscataway, NJ). Due to its large size (3,222 nucleotides), the first half and second halves of the *Igl2* gene were also amplified by PCR separately. During PCR,

nucleotides encoding a SmaI restriction site were added to the 5' end of the gene and nucleotides encoding a XhoI restriction site were added to the 3' end of the gene (see primers in Table 1). The four halves of the two Igl isoforms were subcloned into the 'Trigger' vector (gift from Dr. Upinder Singh, Stanford University School of Medicine, Palo Alto, CA) by restriction enzyme digestion and the Fast-Link DNA Ligation Kit (Epicentre, Madison, WI).

Transfection

E. histolytica trophozoites were transfected with 130 μg of plasmid DNA (encoding shRNA or trigger constructs) by electroporation as previously described [15].

Mutant cell lines transfected with shRNA constructs were maintained in TYI-S-33 medium supplemented with 15 μg/ml hygromycin. The expression of the Igl shRNA was optimized by increasing the hygromycin concentration 15 μg/ml per day until a concentration of 100 μg/ml was reached and was then maintained for 48 hours prior to use in assays. Prior to performance of assay, cells in culture tubes were incubated on ice for 8 minutes and cells in flasks were incubated on ice for 20 minutes. Mutant cells transfected with plasmids for trigger-mediated knock down of expression were maintained at 6 μg/ml G418.

Western blotting

To prepare cell lysates, cells were washed with tris buffered saline (TBS), counted and 1 x 10^5 cells were collected by centrifugation (500 x g for 5 minutes at room temperature). After the cells were resuspended in lysis buffer (Cell Surface Protein Isolation Kit, Pierce, Rockford, IL), the cell lysate was incubated on ice for 30 minutes with vortexing every 5 minutes. The cells were then frozen in liquid nitrogen and thawed in a 37°C water bath three times. Cell lysates were then added to a mixture of NuPAGE LDS sample buffer (Novex, Carlsbad, CA), diluted 1:2 with sterile water and β-mercaptoethanol (10.3% v/v). Proteins were resolved by SDS-PAGE and blotted to PVDF membrane as described [16].

Membranes were blocked by incubation in 5% powdered milk/0.5% Tween 20/TBS for 30 minutes at 37°C and then exposed to primary antibodies: a mixture of monoclonal anti-Lgl antibodies (3C2, IC8, IA9, and ID4) (1:3,333 dilution), a mixture of monoclonal anti-Igl antibodies (3G5-A3-G3, 5H1-F11-D11, and 4G2- D8-H1) (1:3,333 dilution) (both kind gifts from Dr. William A. Petri, Jr., University of Virginia School of Medicine, Charlottesville, VA), polyclonal anti-Hgl antibody (1:5,000) (described above), or monoclonal anti-actin antibody (1:2,500 dilution) (Abcam, Cambridge, MA). The membranes were washed extensively in 0.5% Tween 20/TBS, before incubation with the appropriate peroxidase-conjugated secondary antibody (1:5,000 dilution for goat anti-rabbit; 1:5,000 dilution for goat anti-mouse) (Cappel; ICN Pharmaceuticals, Costa Mesa, CA) for 1 hour at room temperature. Development was achieved by Pierce ECL Western

Blotting Substrate (Thermo Scientific, Waltham, MA) according to the manufacturer's directions. Densitometry was used to measure the intensity of Hgl, Lgl, Igl or actin bands using ImageJ software (version 1.48; U.S. National Institute of Health, Bethesda, MD).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA). To synthesize cDNA, 2μg of total RNA was reverse transcribed using the SuperScript III First Strand Synthesis kit (Invitrogen, Carlsbad, CA). Two micrograms of the resulting cDNA and primers specific for Igl1, Igl2, or ssRNA (control) (see Table 1) was used for amplification over 30 cycles; each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 58.6°C for 30 seconds, and chain elongation with GoTaq DNA Polymerase (Promega, Madison, WI) at 72°C for 30 seconds with a final extension at 72°C for 10 minutes. Generated amplicons were checked by 2% agarose gel electrophoresis and visualized with ethidium bromide [17].

Lipid raft isolation

To stimulate cells, 3.5 x 10^6 trophozoites were incubated in collagen-coated flasks for 20 minutes at 37°C. Detergent-insoluble membrane was isolated from control (unstimulated) and stimulated cells by extraction (30 minutes at 4°C) with ice cold buffer 1 containing 0.5% (v/v) Triton-X100 and protease inhibitors (40 mM sodium pyrophosphate, 0.4 mM dithiothreitol, 0.1 mg of phenylmethylsulfonyl fluoride/ml, 2 mM EDTA, 1 mM EGTA, 3 mM sodium azide, 10 mM Tris-HCl [pH 7.6]). The mixture was then centrifuged

(14,000 x g for 5 min) at 4°C. The Triton-soluble supernatant (TSS) was removed, and the Triton-insoluble pellet (TIP) was resuspended in 80% (wt/v) sucrose in buffer 1. To resolve lipid raft from actin-rich membrane a noncontinuous sucrose gradient was generated by layering equal volumes of 80 (which also contained the TIP), 50, 30, and 10% (wt/wt) sucrose solutions in buffer 1 and centrifuged at 125,000 x g for 17 hours at 4°C in a Beckman Coulter Optima Max-XP ultracentrifuge. The gradient was fractionated (140 µl/fraction) and the fractions were analyzed using SDS-PAGE and Western blotting with antibodies specific to the Hgl and Lgl [11].

Measurement of adhesion to erythrocytes

Parasite-erythrocyte interaction was tested using a protocol previously described [18] with minor modifications. Trophozoites (1 x 10^5) were washed in PBS, centrifuged (200 x g for 5 min at 4°C) with 1 x 10^6 human erythrocytes, and resuspended in 500 µl serum-free *Entamoeba* media. The trophozoites and erythrocytes were incubated on ice for 30 minutes. Following incubation, the media was removed and the cells were gently resuspended with 50µl of the vital stain, Trypan Blue. A hemocytometer was used to microscopically count rosettes (a parasite bound to least three erythrocytes [19]). For each of the five biological replicates, two or three technical replicates were performed.

Hemolysis assay

The hemolytic activity assay was carried out as previously described [20]. Briefly, a total of 1.25 x 10^8 human erythrocytes were incubated for 1 hour at 37°C with 1.25 x 10^6 PBS washed trophozoites (ratio 100:1) in a final volume of 1 ml of PBS. Following

incubation, the cells were centrifuged for 5 minutes at 1200 x g at room temperature and the absorbance (405 nm) of the resultant supernatant was determined using the Synergy HI Hybrid Reader (BioTek, Winooski, VT) to quantify released hemoglobin. For each of the three biological replicates, two technical replicates were performed.

Erythrophagocytosis assay

To measure erythrophagocytosis, an assay was carried out as previously described [21]. Briefly, a total of 2×10^7 human erythrocytes were incubated for 10 minutes at 37°C with 2×10^5 trophozoites (ratio 100:1) that had been washed once with PBS and twice with serum-free media. The cells were collected by centrifugation and the supernatant was removed. The cells were resuspended in water to lyse extracellular (uninternalized) erythrocytes. The cells were collected again by centrifugation, the supernatant was removed, and the pellet of cells was lysed by suspension in 88% (v/v) formic acid. Lysates were transferred to a 24-well plate and the hemoglobin concentration (a measure of the number of internalized red blood cells) was quantified by measuring absorbance at 405 nm as described above. For each of the five biological replicates, two technical replicates were performed.

Statistical analysis

To compare means, statistical analyses were performed using Social Science Statistics' T-Test calculator (http://www.socscistatistics.com/tests/studentttest/) with a one-tailed

test and a 0.05 level of significance. *P* values of less than 0.05 were considered statistically significant.

IV. Results

Inconsistent knock down of the expression of Igl using a short hairpin RNA (shRNA) approach

A standard method of discerning the function of a protein is to knock down its expression and characterize the resulting phenotype in cells. In *E. histolytica*, others have used small hairpin RNAs to reduce expression of proteins including Igl [14]. Therefore, we transfected trophozoites with three episomal shRNA constructs; one targeted to amino acids 272-300 of Igl1 (Igl(272-300)), one targeted to amino acids 1198-1226 of Igl1 and Igl2 (Igl(1198-1226)) and one to GFP (control) [14]. Western blotting with antibodies specific to Igl and actin (load control) and scanning densitometry were used to measure the expression of Igl in the stable transfectants (Figure 2.1). There was inconsistent knock down in the expression of Igl. The greatest knock down in expression was seen at 21 weeks post-transfection but protein level then increased over time.

Characterization of the submembrane distribution of the Gal/GalNAc lectin subunits in shRNA-transfected cell lines

Despite inconsistent knock down of Igl expression, we characterized lipid rafts around the 21 weeks post-transfection to address the question of whether Igl regulated the submembrane distribution of the Hgl/Lgl heterodimer. Lipid rafts were purified by detergent-extraction and sucrose gradient ultracentrifugation of the detergent-resistant

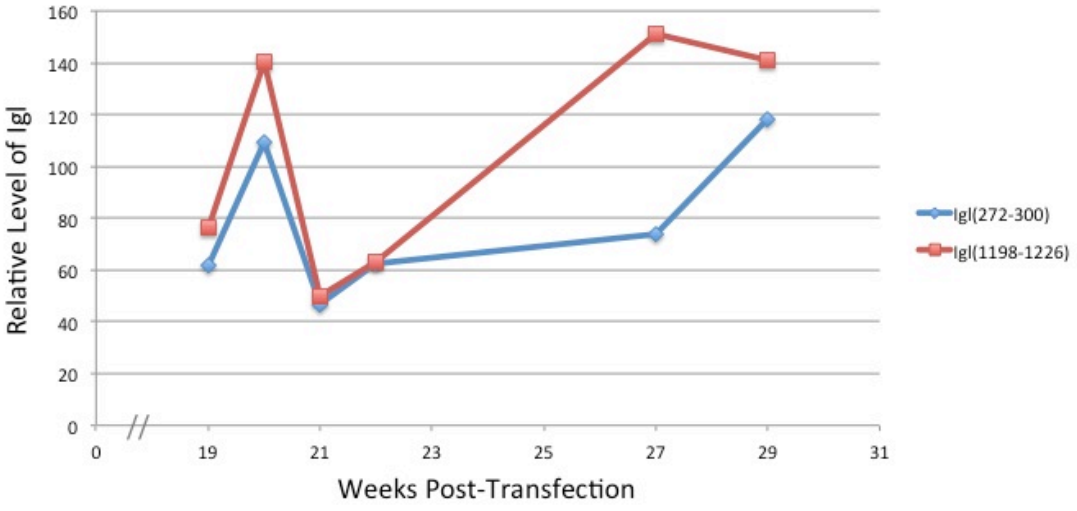

Figure 2.1. Expression of Igl using a short hairpin RNA (shRNA) approach

Scanning densitometry of Western blotting using antibodies specific to Igl in the stable transfectants, Igl(272-300) (blue) and Igl(1198-1226) (red). Western blotting with actin antibodies served as a load control (data not shown). The greatest knock down in expression of Igl was seen 21 weeks post-transfection, after which, expression of Igl increased over time in both cell lines.

membrane. Fractions from the gradient were analyzed using SDS-PAGE and Western blotting with antibodies specific to the Hgl subunit and Lgl subunit. On average, the distribution of the Hgl/Lgl heterodimer appeared to be unchanged in the cell lines expressing Igl-based shRNAs when compared to the GFP control cell line (Figure 2.2A-B). However, in one instance close to 21-weeks post-transfection, when knock down of Igl expression was the greatest, the distribution of the Hgl/Lgl heterodimer appeared to be slightly shifted towards the lighter lipid raft fractions (Figure 2.2C-D). This suggests that Igl may be a negative regulator of Hgl/Lgl-raft interactions. In other words, when levels of Igl are reduced, more Hgl/Lgl tends to interact with raft membrane. However, given the inconsistencies in knock down of Igl, this result could not be confirmed.

Trigger-mediated knock down of Igl expression

Since the application of shRNA technology did not result in a consistent knock down of Igl expression, we attempted to reduce expression of Igl with a different technique known as 'Trigger'. This approach uses a 'Trigger' region to generate small RNAs with sequence specificity to the gene fused to the trigger, in this case Igl, to down-regulate its expression. We subcloned cDNAs encoding four halves of the two Igl isoforms into separate episomal 'Trigger' vectors. These were termed Igl1A (first half of Igl1 sequence in the 'Trigger' vector) (Figure 2.3A), Igl1B (second half of Igl1 sequence in the 'Trigger' vector) (Figure 2.3B), Igl2A (first half of Igl2 sequence in the 'Trigger' vector) (Figure 2.3C), and Igl2B (second half of Igl2 in the 'Trigger' vector) (Figure 2.3D).

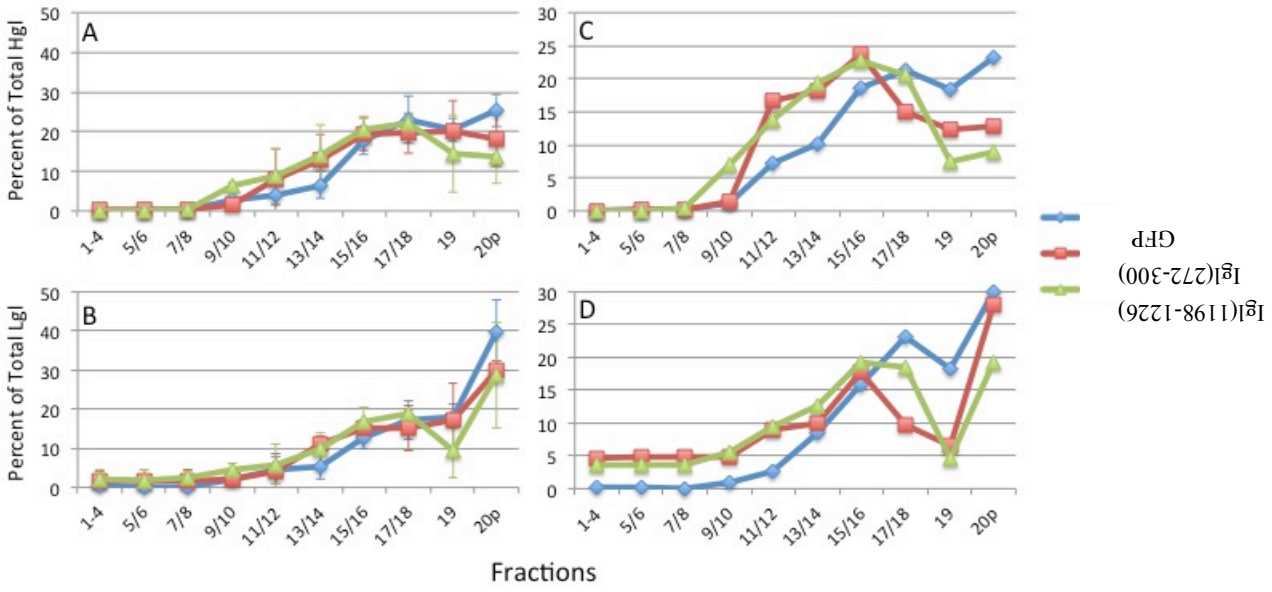

Figure 2.2. Submembrane distribution of the Gal/GalNAc lectin subunits in shRNA-transfected cell lines

Lipid rafts were purified from all cell lines, GFP control (blue), Igl(272-300) (red), and Igl(1198-1226) (green) by detergent-extraction and sucrose gradient ultracentrifugation of the detergent-resistant membrane. Fractions from the gradient were analyzed using SDS-PAGE and Western blotting with antibodies specific to the Hgl subunit (A and C) and Lgl subunit (B and D). Lipid rafts are found in fraction 9/10 [11]. The data in graphs A and B represent the mean ± standard deviation of three trials. There is no change in the distribution of Hgl or Lgl in the transfected cells. The data in graphs C and D represent one trial around 21 weeks post-transfection. The Hgl/Lgl heterodimer appears to be unexpectedly enriched in lighter raft fractions.

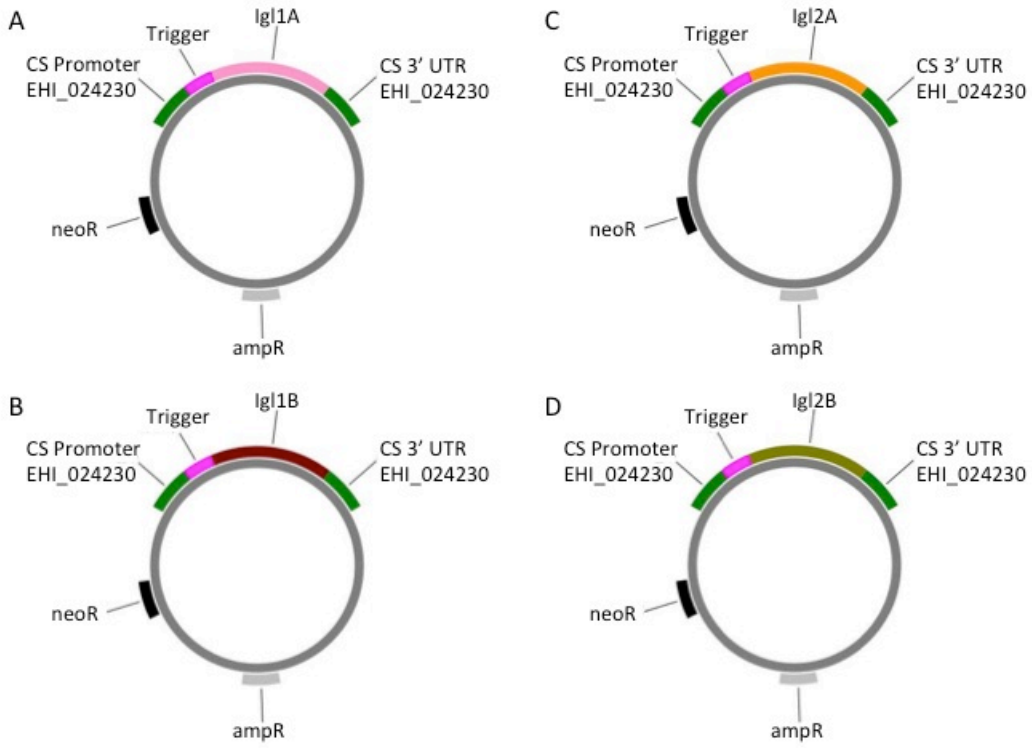

Figure 2.3. 'Trigger' vectors constructed to knock down expression of Igl

The vector uses a 'Trigger' region (hot pink) to generate antisense RNA with sequence specificity to a gene downstream to down-regulate its expression. We subcloned cDNAs encoding four halves of the two Igl isoforms into the 'Trigger' vector. These were termed Igl1A (first half of Igl1 sequence in the 'Trigger' vector) (A), Igl1B (second half of Igl1 sequence in the 'Trigger' vector) (B), Igl2A (first half of Igl2 sequence in the 'Trigger' vector) (C), and Igl2B (second half of Igl2 in the 'Trigger' vector) (D). These plasmids were transfected into wild type *E. histolytica* and stable transfectants were selected for with G418. Plasmid maps were generated by PlasMapper [24].

These plasmids were transfected into wild type *E. histolytica* and stable transfectants were selected for with G418. Expression of Igl was characterized by Western blotting and scanning densitometry as described above.

Inconsistent knock down of the expression of Igl in singly-transfected 'Trigger' cell lines

Cell lines Igl1A-A and Igl1A-B are two independent stable transfectants that harbor the Igl1A-Trigger plasmid. Cell lines Igl1B-A and Igl1B-C are two independent stable transfectants that harbor the Igl1B-Trigger plasmid. Neither of these cell lines exhibited knock down in the expression of Igl when compared to wild type cells (Figure 2.4) or another control cell line that possessed the Trigger vector fused to luciferase (irrelevant protein) (data not shown). Scanning densitometry indicated that the Igl protein was actually being overexpressed in these cell lines (Figure 2.4). One possible explanation for this observation is that knock down of Igl1 is inducing up-regulation of Igl2. Given the high sequence homology between the two isoforms of Igl it is unclear if our antibodies recognize Igl1, Igl2 or both.

Double transfections reveal that Igl2, but not Igl1, can be knocked down in *E. histolytica*

Given the unusual result with Western blotting, and the possible opposing expression patterns of Igl1 and Igl2, we attempted to knock down both isoforms of Igl in a single cell line. Others have shown that the knock down phenotype persists in *E. histolytica* even after removal of selection and loss or "cure" of the plasmid [22]. Furthermore, it has been

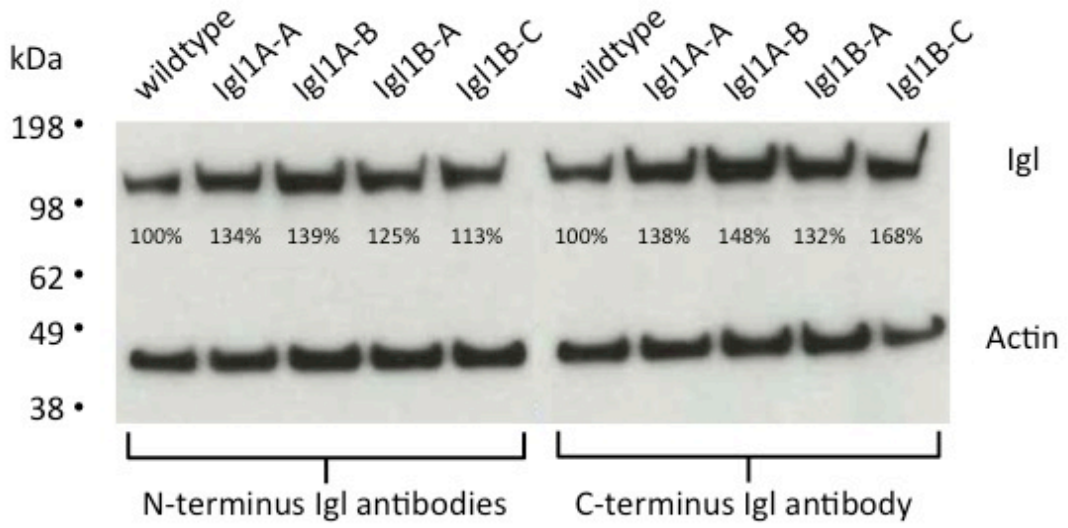

Figure 2.4. Western blot showing inconsistent knock down of the expression of Igl in singly-transfected 'Trigger' cell lines

Cell lines Igl1A-A and Igl1A-B are two independent stable transfectants that harbor the Igl1A-Trigger plasmid (Figure 2.3A). Cell lines Igl1B-A and Igl1B-C are two independent stable transfectants that harbor the Igl1B-Trigger plasmid (Figure 2.3B). The Western blot with antibodies specific to Igl and actin (load control) shown above, six weeks post-transfection, indicates neither of these cell lines exhibited knock down in the expression of Igl when compared to wild type cells. Percent expression of Igl, corrected for load and compared to wild type control, shown under representative band.

shown that a second Trigger plasmid can be introduced into cured cell lines resulting in the Trigger-mediated knock down of a second protein [23]. Trophozoites harboring Igl1A-Trigger or Igl1B-Trigger were taken out of selection for 12 weeks. These cured cell lines were transfected with Trigger-plasmids possessing sequence targeting Igl2. We obtained one stable transfectant, 1A2A, which was presumptively knocked down Igl expression with sequences targeting the first half of Igl1 and the first half of Igl2.

We chose to measure expression of Igl, in the 1A2A cell line using reverse transcriptase polymerase chain reaction (RT-PCR) (Figure 2.5), to overcome the problem of antibody specificity. Here it was possible to distinguish between Igl1 and Igl2 message with specific primers. In particular, we used one set of primers specific to Igl1, two sets of primers specific for Igl2, and primers for ssRNA (control). Compared to a control cell line possessing a Trigger vector fused to luciferase (Luc), the 1A2A cell line showed no Igl1 knock down, but on average, ~57% knock down in Igl2 (Figure 2.5). Although substantial, the knock down was not quite statistically significant. However, the reduction is approaching significance.

Knock down of Igl2 does not affect adhesion or hemolysis, but slightly inhibits erythrophagocytosis

Since we observed knock down of Igl2, we wanted to test several virulence functions to see if the knock down affected pathogenicity of the parasite. We used a rosette assay to test the ability of the transgenic cell line to adhere to human erythrocytes. Parasites and

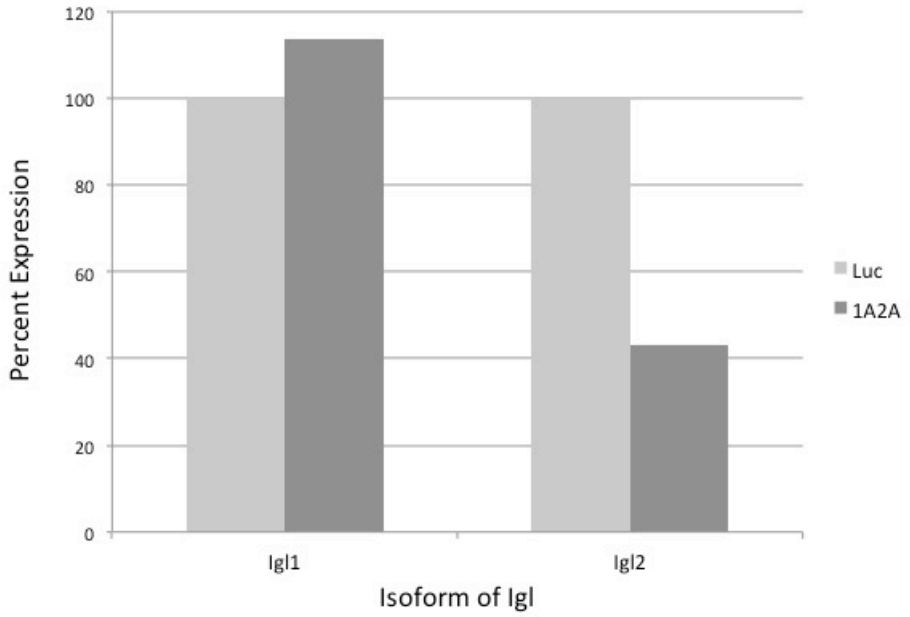

Figure 2.5. RT-PCR analysis of Igl1 and Igl2 in doubly-transfected 'Trigger' cell lines

Four sets of primers were used (see Table S1); one set of primers specific for Igl1, two sets of primers specific for different regions of Igl2 (set 1 and set 2), and one set of primers specific for ssRNA, used as loading control. Results from scanning densitometry of DNA bands of two biological replicates was performed showing the percent of Igl1 or Igl2 message in mutant cells standardized to the Luc control cell line (arbitrarily set to 100%) and ssRNA. The 1A2A cell line showed no Igl1 knockdown, but on average, ~57% knock down in Igl2. The knock down of Igl2 is approaching significance (p=0.092289).

erythrocytes were incubated together on ice. After 30 minutes, the number of parasites bound to at least three erythrocytes (a rosette) was counted manually by microscopy. The number of rosettes formed by the 1A2A cell line was slightly lower than that of the Luc control, albeit the difference was not statistically significant (Figure 2.6). It would appear that the knock down in expression of Igl2 does not inhibit the trophozoite from adhering to erythrocytes.

Next, a hemolysis assay was performed. Trophozoites and host erythrocytes were incubated together for one hour at 37°C. The amount of released hemoglobin from lysed erythrocytes (as an indicator of host cell lysis) was quantified by spectrophotometry. There was no difference in the hemolysis capability of the 1A2A cell line compared to that in the Luc control (Figure 2.7), suggesting Igl2 does not play a role in hemolysis.

A phagocytosis assay was also performed. Trophozoites were incubated with erythrocytes for 10 minutes at 37°C. Cells were resuspended in water to lyse the uninternalized erythrocytes and then collected by centrifugation. The supernatant was removed and the cells were lysed with formic acid. Hemoglobin concentration was measured using a spectrophotometer. On average, there was a slight defect in erythrophagocytosis (~17%) in the 1A2A cell line compared to the Luc control, which was determined to be statistically significant (Figure 2.8). This suggests that Igl2 might play a role in phagocytosis.

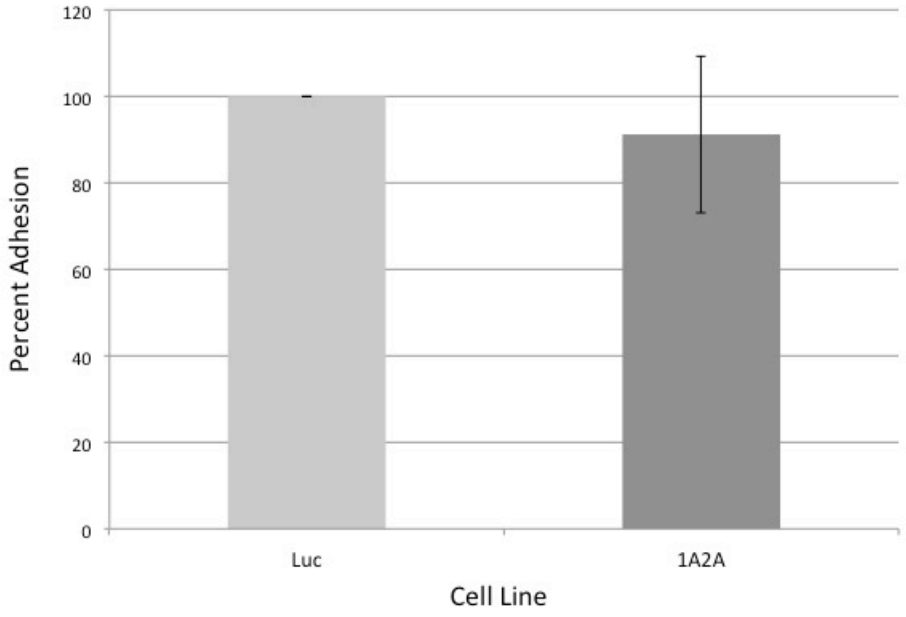

Figure 2.6. Analysis of parasite-erythrocyte adhesion of 1A2A cell line compared to Luc control

Parasites and erythrocytes were incubated together on ice. After 30 minutes, the number of parasites bound to at least three erythrocytes (a rosette) was counted manually by microscopy. The number of rosettes formed by the 1A2A cell line was slightly lower than that of the Luc control (arbitrarily set to 100%), but the difference was not statistically significant (n=5), indicating that the knock down in expression of Igl2 does not inhibit trophozoites from adhering to erythrocytes.

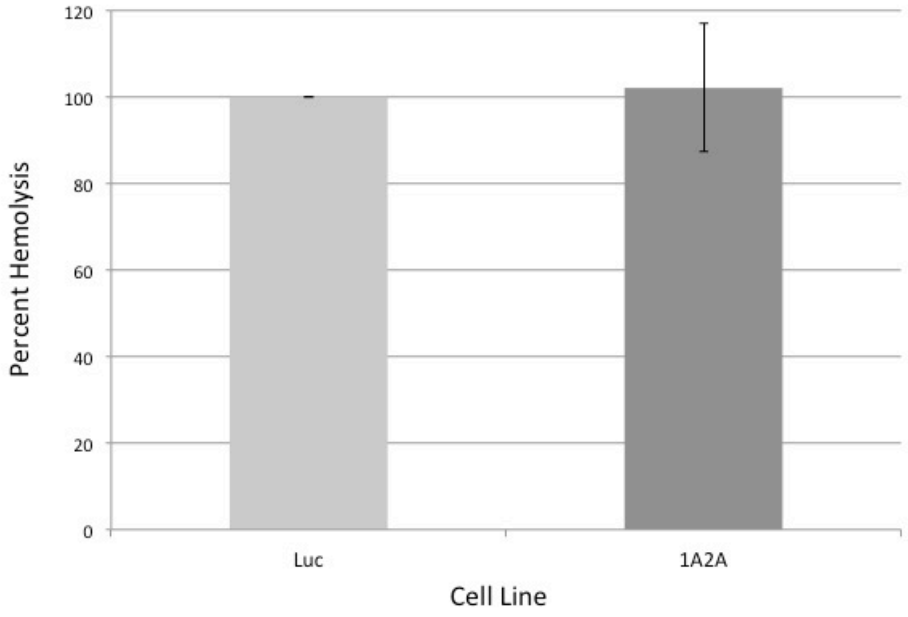

Figure 2.7. Analysis of hemolytic activity of 1A2A cell line compared to Luc control

Trophozoites and host erythrocytes were incubated together for one hour at 37°C. The amount of released hemoglobin (as an indicator of host cell lysis) was quantified by spectrophotometry. There was no difference in the hemolysis capability of the 1A2A cell line compared to that in the Luc control (n=3), suggesting Igl2 does not play a role in hemolysis.

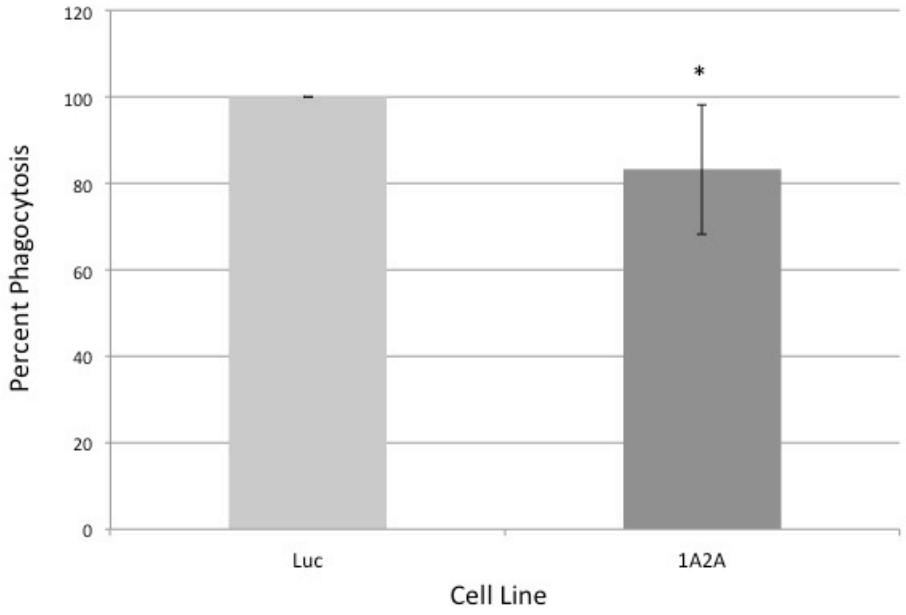

Figure 2.8. Analysis of erythrophagocytosis by 1A2A cell line compared to Luc control

Trophozoites were incubated with erythrocytes for 10 minutes at 37°C. Cells were resuspended in water to lyse the uninternalized erythrocytes and then collected by centrifugation. The supernatant was removed and the cells were lysed with formic acid. Hemoglobin concentration was measured using a spectrophotometer. On average, there was a slight defect in erythrophagocytosis (~17%) in the 1A2A cell line compared to the Luc control (n=5), which was determined to be statistically significant (p=0.018873). This suggests that Igl2 might play a role in phagocytosis.

The 1A2A cell line possesses a typical submembrane distribution of the the Hgl/Lgl heterodimer

In addition to the virulence assays performed, we wanted to characterize the submembrane distribution of the Hgl/Lgl heterodimer in the 1A2A cell line. As described above, lipid rafts were isolated by detergent-extraction and sucrose gradient density ultracentrifugation of the detergent-resistant membrane. Fractions were analyzed using SDS-PAGE and Western blotting with antibodies specific to the Hgl subunit and Lgl subunit. The submembrane distribution of the Hgl/Lgl heterodimer in the 1A2A cell line was identical to that in the Luc control cell line (Figure 2.9). This suggests that unlike Igl1 (Figure 2.2), Igl2 does not regulate the submembrane distribution of the Hgl/Lgl heterodimer.

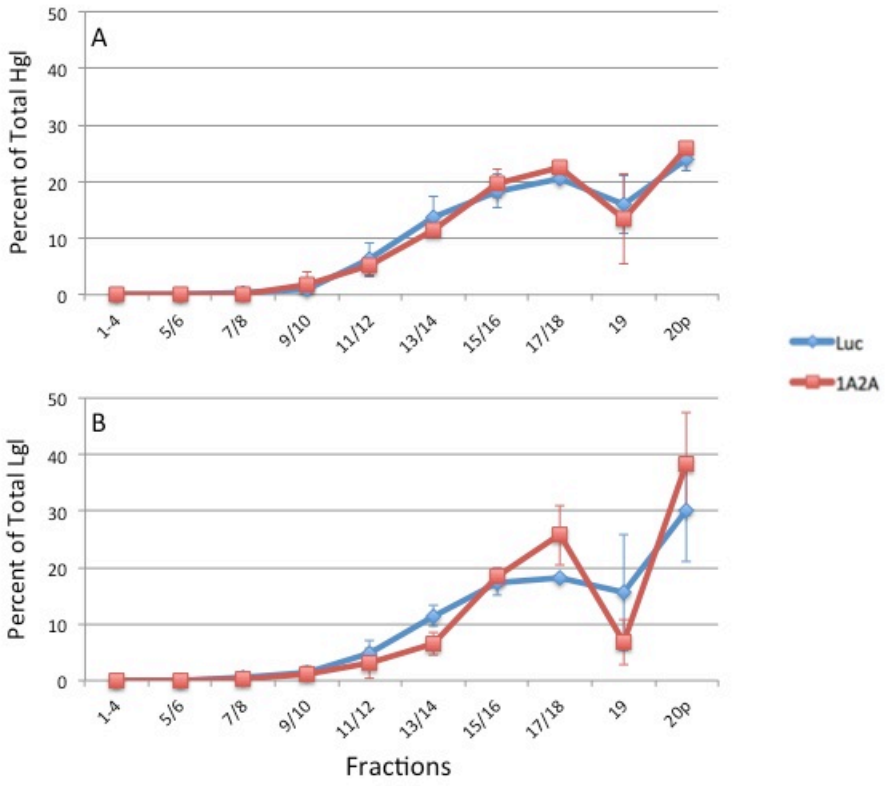

Figure 2.9. Submembrane distribution of the Gal/GalNAc lectin subunits in doubly-transfected 'Trigger' cell line, 1A2A

Lipid rafts were isolated from the Luc control cell line (blue) and the 1A2A cell line (red) by detergent-extraction and sucrose gradient ultracentrifugation of the detergent-resistant membrane. Fractions were analyzed using SDS-PAGE and Western blotting with antibodies specific to the Hgl subunit (A) and Lgl subunit (B). The submembrane distribution of the Hgl/Lgl heterodimer in the 1A2A cell line was typical, and was identical to that in the Luc control cell line, suggesting that Igl2 does not regulate the submembrane distribution of the Hgl/Lgl heterodimer.

V. Discussion

In this study we have shown that it may not be possible to knock down the expression of Igl1, one of the isoforms encoding an intermediate subunit of the Gal/GalNAc lectin, long term. This result was obtained using two different approaches to reduce expression of the protein. We have also shown that it is possible to knock down expression of the second isoform, Igl2, and that this isoform may be involved in erythrophagocytosis. The Gal/GalNAc lectin mediates parasite adhesion to host cells and host extracellular matrix, which is necessary for invasion and pathogenicity [1].

The first attempt to knock down expression of Igl employed an shRNA approach. A cell line with persistently low expression of Igl could not be obtained. In fact, SDS-PAGE and Western blotting revealed an increase in the total level of Igl protein over time. Given that our antibodies may not be specific to one isoform or the other, the increase may be due to higher levels of Igl1, Igl2 or both. One possible explanation is knock down of Igl1 causes the cells to up-regulate expression of Igl2 to compensate for the loss of the other isoform. A similar pattern was seen in cell lines in which one or more of the Lgl subunits were silenced. There are five isoforms of Lgl that can be sorted into two groups: Lgl1-3 in one group and Lgl4-5 in another group. When Lgl1 was silenced, there was simultaneous silencing of Lgl 2 and Lgl3, and the Lgl4 and Lgl5 isoforms were consequently up-regulated. Alternatively, when Lgl5 was silenced, which simultaneously silences Lgl4, the other group of isoforms, Lgl1-3, were up-regulated [25]. Another explanation is that the knock down of Igl1 is lethal to cells and, therefore, a stable knock

down of Igl cannot be cultured. The only cells surviving are those that are resistant to selection (possess the plasmid), but in some way have acquired a compensatory mutation that prevents the knock down of Igl. This has also been suggested for Lgl. Silencing of Lgl1-3 was achieved in one cell line, and silencing of Lgl4-5 was achieved in another cell line, but attempts to simultaneously silence all five Lgl genes were unsuccessful, suggesting the cell cannot survive without the Lgl subunit [25]. Since we could not knock down Igl expression, it is possible that Igl has some other essential function other than adhesion.

Considering the important role the Gal/GalNAc lectin plays in adhesion, it would not be surprising that complete silencing of any one subunit of the lectin would be lethal. While characterizing the submembrane distribution of the Hgl/Lgl heterodimer in the shRNA-transfected cell lines, an unexpected enrichment of the heterodimer towards the lighter lipid raft fractions was seen, and this occurred around the same time that the knock down in expression of Igl was the greatest (~21 weeks post-transfection). The observation that the Hgl/Lgl heterodimer becomes enriched in rafts when Igl levels are decreased suggests that Igl may be a negative regulator of Hgl/Lgl-raft interactions. However, confirmation of this hypothesis was not possible because of the inconsistent level of knock down in the shRNA-transfected cell lines. Overall, this idea does not support the notion that Igl serves as an anchor for the Hgl/Lgl heterodimer in rafts.

We used the Trigger approach as an alternative to shRNA and as a means to knock down both isoforms of Igl. Specifically, cells were transfected with the Trigger vector harboring sequences targeting two different domains (5' or 3') of Igl1. Stable transfectants were subsequently relieved of selection and transfected with a second plasmid harboring sequences specific for Igl2. Others have shown that the removal of selection in cell lines containing 'Trigger' plasmids causes these cells to lose their plasmids, but, by an unknown mechanism, retain the knock down phenotype [22]. RT-PCR analysis revealed that the doubly transfected cell line (1A2A) possessed normal Igl1 expression but reduced Igl2 expression, with reduction approaching significance. It is important to note that the 'Trigger'-mediated knock down of genes is not always successful. Others have found that certain RNAi machinery genes cannot be silenced using the 'Trigger approach', even though functional antisense small RNAs to the genes of interest are being generated. An explanation provided for this result was that these genes could not be silenced because they are essential for the survival of the parasite [26]. This same line of thinking may also be true for Igl1. It remains to be seen if Igl2 can be silenced simply by transfecting cells with the Trigger plasmid containing Igl2-specific sequences.

Characterization of the virulence potential of the 1A2A cell line revealed that the down-regulation of Igl2 does not affect two important virulence functions, adhesion to or lysis of host cells. This is in contrast to a previous study that found that recombinant Igl1 has hemolytic activity [10]. One possibility is that Igl1 regulates hemolysis while Igl2 does

not. If this is the case, Igl1 would be more important for virulence than Igl2. In support of this, it has been shown that the levels of Igl2 are comparable in *E. histolytica* and non-pathogenic *E. dispar*, while the levels of Igl1 are higher in the pathogenic *E. histolytica* [27]. It is also conceivable that the recombinant fragments of Igl1, a protein normally bound to the membrane, interacted non-specifically with the erythrocyte membranes. Thus, the *in vitro* lysis of red blood by recombinant Igl1 may be an artifact of the system.

Our results are also in contrast with a study that found three mAbs specific to Igl inhibit trophozoite adhesion to CHO cells. One of these antibodies was specific to Igl1, one antibody was specific to Igl2, and one antibody was specific to both isoforms, but all three were able to inhibit adhesion, suggesting both Igls are involved in the process [28]. However, we saw no adhesion defect in the cell line that showed a knock down of Igl2. Perhaps the knock down of the Igl2 gene (~57% on average) was not enough to affect the parasite's ability to adhere. Another difference is that we used host erythrocytes instead of CHO cells and the mechanism of adhesion to different host cells may vary. Virulence testing of the 1A2A cell line also indicated a significant reduction in the parasites' ability to phagocytose erythrocytes. This is the first study to show that the down-regulation in expression of Igl2 may inhibit erythrophagocytosis.

The submembrane distribution of the Hgl/Lgl heterodimer was also characterized for the 1A2A cell line using detergent-extraction and sucrose gradient density ultracentrifugation of the detergent-resistant membrane to isolate and purify lipid rafts. The unstimulated

cells showed an expected distribution of the Hgl/Lgl heterodimer, with little to no enrichment in the lipid raft fractions. Thus, Igl2 may not regulate the submembrane position of Hgl/Lgl. Previous studies have shown that incubation with host cells or host extracellular matrix induces enrichment of Hgl/Lgl in lipid rafts [many from our lab]. Similar experiments could not be done with the 1A2A cells line because the lipid raft-enriched phenotype could not be obtained even for wildtype cells (data not shown). Thus, it remains to be seen if stimulation can alter the submembrane distribution of the Gal/GalNAc lectin subunits.

In summary, it would appear that the expression of Igl1 cannot be knocked down, because it may be essential for parasite viability and pathogenicity. Knock down in the expression of Igl2 causes a slight defect in erythrophagocytosis, but no other phenotype has been determined. Although the Gal/GalNAc lectin is the most well-characterized amoebic cell surface receptor, there is still much insight to be gained about the assembly of the three subunits that make up this active protein. Future studies may seek to determine if Igl1 plays a more important role in virulence than Igl2, and what that role is, specifically. These studies could use a cell line overexpressing Igl1, since a stable, long term knock down of this isoform may not be possible. The examination of the submembrane distribution of the Hgl/Lgl heterodimer in stimulated trophozoites that have an Igl knock down phenotype and in trophozoites that have been singly transfected with an Igl2-Trigger vector could also shed light on the assembly of the Gal/GalNAc lectin into an active complex.

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VII. Supplemental Material

Table S1. Primers for construct cloning and RT-PCR

All primers used in this study to clone Igl sequences into the 'Trigger' vector and to measure expression of Igl by RT-PCR are listed below.

Cloning Primers	**Sequence (5' to 3')**
Igl1A Forward	CCCCCGGGATGTTTATTCTTCTTTTATTC
Igl1A Reverse	CCCTCGAGGTCAAAATAATGTGCATC
Igl1B Forward	CCCCCGGGATGCACATTATTTTGAC
Igl1B Reverse	CCCCTCGAGTTAGAACATAAATGCTAAC
Igl2A Forward	CCCCCGGGATGTTTATTCTTCTTTTATTC
Igl2A Reverse	CCCTCGAGCTGGACATTCTTGAC
Igl2B Forward	CCCCCGGGGTCAAGAATGTCCAG
Igl2B Reverse	CCCCTCGAGTTAGAACATAAATGCTAACATG
RT-PCR Primers	**Sequence (5' to 3')**
Igl1 Forward	CATCATCACACTCTGGAAATGATAAG
Igl1 Reverse	CCATCAACAGTAGTAGACATCCC
Igl2, set 1 Forward	CTTGCTGCTAGTGGTTCAAATG
Igl2, set 1 Reverse	TGCAGGACATGGACTACAATAC
Igl2, set 2 Forward	TGCCCAGTGTGCTTCTAATG
Igl2, set 2 Reverse	GCTGAACATGCTGAACAATGTC
Eh ssRNA Forward	AGGCGCGTAAATTACCCACTTTCG
Eh ssRNA Reverse	CACCAGACTTGCCCTCCAATTGAT